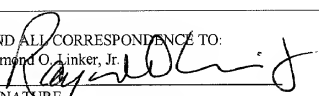
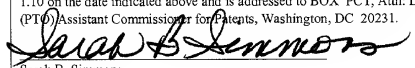


FORM PTO-1599 (REV. 10-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 33339/206076
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) To be assigned 09/700687
INTERNATIONAL APPLICATION NO. PCT/FR99/01165	INTERNATIONAL FILING DATE May 17, 1999	PRIORITY DATE CLAIMED May 22, 1998	
TITLE OF INVENTION MUTANT LACTOBACILLUS BULGARICUS STRAINS FREE FROM BETA-GALACTOSIDE			
APPLICANT(S) FOR DO/EO/US Laurent BENBADIS; Pierre BRIGNON; François GENDRE			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11. To 16. Below concern other document(s) or information included:</p> <p>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: Statement in Support of Filing a Sequence Listing, printed sequence listing, computer readable disk.</p>			

U.S. APPLICATION NO. (If known, see PCT Art. 17) To be assigned 09/700687	INTERNATIONAL APPLICATION NO. PCT/FR99/01165	ATTORNEY'S DOCKET NUMBER 33359/206076
17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO \$690.00 But all claims did not satisfy provisions of PCT Article 33(1)-(4) International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$ 860.00
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$
CLAIMS	NUMBER FILED	NUMBER EXTRA
Total Claims	10 -20 =	0
Independent Claims	1 - 3 =	0
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		+ \$270.00
TOTAL OF ABOVE CALCULATIONS =		\$ 860.00
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by one-half.		\$
SUBTOTAL =		\$
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		\$
TOTAL NATIONAL FEE =		\$ 860.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +		\$
TOTAL FEES ENCLOSED =		\$ 860.00
		Amount to be Refunded \$ Charged \$
a. <input checked="" type="checkbox"/> A check in the amount of \$ 860.00 to cover the above fees is enclosed.		
b. <input type="checkbox"/> Please charge my Deposit Account No. 16-0605 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.		
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605.		
Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.		
SEND ALL CORRESPONDENCE TO: Raymond O. Linker, Jr.  SIGNATURE REGISTRATION NUMBER 26,419 ALSTON & BIRD LLP Post Office Drawer 34009 Charlotte, NC 28234 Tel. Charlotte Office (704) 331-6000 Fax Charlotte Office (704) 334-2014 Customer Number 000826		"Express Mail" Mailing Label Number EL 432822785 US Date of Deposit: November 17, 2000 I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to BOX PCT, Attn: DO/US (PTO) Assistant Commissioner for Patents, Washington, DC 20231.  Sarah B. Simmons

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re: Laurent Benbadis et al. Attn: DO/US
International Appl. No.: PCT/FR99/01165
International Filing Date: May 17, 1999
For: MUTANT LACTOBACILLUS BULGARICUS
STRAINS FREE FROM BETA-GALACTOSIDE
ACTIVITY

November 17, 2000

Box PCT
Assistant Commissioner of Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified application as follows:

In The Abstract:

Please add the following as page 16 of the application:

ABSTRACT

The invention concerns mutant *L. bulgaricus* strains bearing a nonsense mutation, in at least one of the sequences coding for the lactose operon, and free from β -galactosidase activity, and lactic starters comprising said strains. Said strains and starters can be used to obtain fermented milk products from glucose-added milk.

In The Claims:

Claim 3, line 2, please delete "either of claims 1 and 2" and substitute --claim 1--.

Claim 4, line 3, please delete "any one of claims 1 to 3" and substitute --claim 1--.

Claim 6, line 5, please delete "any one of claims 1 to 3" and substitute --claim 1--.

Claim 8, lines 1 and 2, please delete "either of claims 6 and 7" and substitute --claim 6 --.

Claim 9, line 2, please delete "any one of claims 6 to 8" and substitute --claim 6--.

REMARKS

The above amendments are made to more clearly define the invention under United States practice. Please enter this amendment prior to calculation of the filing fee.

Respectfully submitted,



Raymond O. Linker, Jr.
Registration No. 26,419

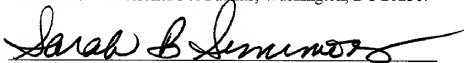
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Sarah B. Simmons

CLT01/4452905v1

WO 99/61627

1

MUTANT LACTOBACILLUS BULGARICUS STRAINS FREEFROM BETA-GALACTOSIDASE ACTIVITY

These strains and ferments can be used for obtaining fermented dairy products from milk supplemented with glucose.

The present invention relates to novel variants of *bulgaricus* and to their use for preparing fermented dairy products.

Yogurts are conventionally obtained by fermentation of milk with a combination of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. During the fermentation, which is carried out at a temperature of approximately 40 to 45°C, these bacteria use mainly lactose as an energetic substrate, and produce lactic acid which causes the milk to coagulate; when the pH reaches a value of approximately 4.8 to 4.5, this fermentation step (also named "acidification") is terminated by cooling the product. This product is then kept in the cold during the remainder of the manufacturing and packaging process, and until its consumption.

However, the cooling does not completely stop the lactic acid fermentation; even when the product is kept at 4°C, a gradual increase in its acidity is observed over time.

This phenomenon, known as postacidification, is responsible for degradation of the organoleptic qualities of the product during its conservation.

The postacidification results essentially from the use by the bacteria, and mainly by *L. bulgaricus*, of the lactose remaining in the product at the end of the controlled acidification step. In order to avoid it, it has been proposed to use strains of *L. bulgaricus* which ferment lactose hardly or not at all.

One of the enzymes which are essential for the fermentation of lactose is β -galactosidase, which hydrolyzes lactose into glucose and galactose. It has

therefore been proposed, in order to obtain non-postacidifying strains of *L. bulgaricus*, to produce artificial mutants, or to select natural mutants, in which the activity of this enzyme is affected.

5 For example, patent EP 402 450 in the name of GENENCOR describes the production, by localized mutagenesis of the β -galactosidase gene, of conditional mutants of *L. bulgaricus*, in which the β -galactosidase, which is active during the fermentation at 40°C, loses
10 its activity at the temperature or at the pH corresponding to the conditions of conservation of fermented dairy products.

Application JP 90053437 describes the production of an artificial mutant of *L. bulgaricus*
15 which has completely lost the capacity to ferment lactose, and the selection of a natural mutant with decreased lactose fermentation capacity; these mutants are however both capable of developing and acidifying normally in the presence of *S. thermophilus*, on
20 condition that the medium is supplemented with glucose. The subcultures of these mutants conserve their acidification characteristics, in milk lacking glucose, after 10 subculturings.

Patent EP 0518 096, in the name of the SOCIÉTÉ
25 DES PRODUITS NESTLÉ, proposes to use, for manufacturing yogurt, poorly postacidifying mutants of *Lactobacillus bulgaricus* which have been preselected on the criterion of the deletion of a fragment of the β -galactosidase gene. The screening and characterization of these
30 mutants are facilitated due to the fact that the presence of this deletion can be easily verified on restriction profiles. In addition, the deletions are known to be irreversible mutations, which makes it possible to easily obtain stable mutant strains from
35 the parent strain. Patent EP 0518 096 describes two types of weakly postacidifying mutants selected in this way. The first have a deletion which affects only the β -galactosidase gene; when they are combined with *S. thermophilus* and cultured on milk, they exhibit,

even without the addition of glucose, growth and acidification properties which are comparable to those of the wild-type strain from which they are derived. The second have a larger deletion, stretching over at least 1 kb downstream of the β -galactosidase gene; when they are combined with *S. thermophilus*, they grow more slowly and acidify much less than the wild-type strain from which they are derived; the addition of glucose to the culture medium has only a slight influence on their acidification and postacidification properties.

Natural mutants in which the β -galactosidase is inactive are much more difficult to select and to maintain as pure cultures in the case of point mutations than in the case of deletion mutants; this is explained by the lower probability of a point mutation producing an inactive protein, by the greater difficulty in localizing and characterizing the point mutations using restriction profiles, and by the very high reversion rate.

The applicant has now found other natural mutants of *L. bulgaricus*, which do not carry a deletion in the gene encoding β -galactosidase, and which have advantageous technological characteristics. In the context of the present invention, a non-sense mutant, which is incapable of assimilating lactose, has been isolated from a culture of a wild-type *L. bulgaricus*. When combined with *S. thermophilus*, in culture on milk, it grows and acidifies much more slowly than the wild-type strain from which it is derived. Conversely, its growth and its acidification are virtually normal when the milk is supplemented with glucose.

A subject of the present invention is a mutant strain of *L. bulgaricus* lacking β -galactosidase activity, characterized in that it carries a mutation which introduces a non-sense codon into one of the coding sequences of the lactose operon, and in particular the sequence encoding β -galactosidase.

A strain of *L. bulgaricus* in accordance with the invention was deposited according to the Treaty of

Budapest, on January 14, 1998, with the CNCM (Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures]) held by the Pasteur Institute, 25 rue du Docteur Roux, in Paris, under the number I-1968.

This strain has the following morphological and biochemical characteristics:

- Morphology: Gram-positive microorganism, immobile, isolated or short-chain, asporogenic, pleomorphic, thin bacilli.
- Metabolism: homofermentative, catalase (-).
- Fermentation of sugars: D-glucose (+), D-fructose (+), D-mannose (+), esculine (+).

The inventors have sequenced the lactose operon in the I-1968 mutant. The corresponding sequence is represented in the appended sequence listing under the number SEQ ID No: 1. The sequences of the translation products (permease and β -galactosidase) are represented under the numbers SEQ ID No: 2 and SEQ ID No: 3, respectively.

The analysis of this sequence reveals two point mutations: one, in the permease gene (position 122 of the sequence SEQ ID No: 1), induces an amino acid change (Lys \rightarrow Asn); the other, in the β -galactosidase gene (position 4519 of the sequence SEQ ID No: 1), introduces a stop codon. Although conserving its active sites (positions 464 and 531), the β -galactosidase produced by this mutant is inactive. The inventors have also noted that this mutation remains stable after several series of subculturing, on a culture medium containing glucose. On the other hand, on a culture medium without glucose, this non-sense mutation reverts very rapidly at a rate of approximately 10^{-6} .

The present invention also encompasses mutant strains which are incapable of assimilating lactose and which are derived from the I-1968 strain. Such strains can, for example, be obtained by inducing other mutations in the lactose operon of the I-1968 strain, by site-directed mutagenesis.

A subject of the present invention is also a lactic ferment, in particular a yogurt ferment, characterized in that it comprises at least one strain of *L. bulgaricus* in accordance with the invention as defined above, preferably combined with at least one strain of *S. thermophilus*.

For the production of a ferment in accordance with the invention, any strain of *S. thermophilus* which is suitable for manufacturing yogurt can be used; the choice of one or more strains of *S. thermophilus* can be made as a function of the additional characteristics that it is desired optionally to confer on the finished product.

By way of example of strains of *S. thermophilus* which can be used in combination with a strain of *L. bulgaricus* in accordance with the invention, mention may be made of the following strains, deposited with the CNCM (Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures]) held by the Pasteur Institute, 25 rue du Docteur Roux, in Paris:

- the strain deposited on August 25, 1994, under the number I-1470, and the strain deposited on August 23, 1995, under the number I-1620; these two strains are described in the European Application published under the number 96/06924;

- the strains deposited on December 30, 1994, under the numbers I-1520 and I-1521; these 2 strains are described in PCT international application WO 96/20607;

- the strain deposited on October 24, 1995 under the number I-1630; the characteristics of this strain are described in PCT international application WO 96/01701.

These strains can be combined mutually or with one or more other industrial strains of *S. thermophilus*.

The strain(s) of *S. thermophilus* is (are) combined with the strain(s) of *L. bulgaricus* in

accordance with the invention, in the same way and in the same proportions as in conventional yogurt ferments; the population of *L. bulgaricus* bacteria in accordance with the invention may, for example, represent between 10 and 90%, preferably between 20 and 50%, of the total bacterial population.

A subject of the present invention is also a method for preparing a fermented dairy product, characterized in that it comprises a step during which milk is fermented using a ferment comprising at least one strain of *L. bulgaricus* in accordance with the invention, in the presence of at least one sugar which can be assimilated by said strain; it can be in particular fructose, mannose and, preferably, glucose. Advantageously, said fermented dairy product is a yogurt.

The method in accordance with the invention is similar to conventional methods for preparing yogurt with regard to the main methods of implementation of the controlled acidification step; in particular, this acidification is carried out at a temperature of between 20 and 45°C, and preferably between 30 and 45°C, and "batchwise", i.e. in a single step and using a single fermentation tank.

The duration of this controlled acidification step is generally about 6 to 24 hours, and preferably about 6 to 16 hours; it is therefore longer than in the case of conventional methods for preparing yogurt (in which it is 3 to 5 hours at 44°C). Specifically, the strains of *L. bulgaricus* in accordance with the invention, even combined with *S. thermophilus*, grow and acidify much more slowly than the wild-type strains.

In addition, the rate of growth and acidification of the strains of *L. bulgaricus* in accordance with the invention varies very significantly depending on the amount of glucose added to the milk. This property makes it possible to control their growth and their acidification, by simply adding the desired amount of glucose at the start of fermentation.

The inventors have also observed that, when strains of *L. bulgaricus* or ferments in accordance with the invention are used, the acidification slows down considerably when the pH reaches the range of 4.8 to 4.5 (which corresponds to the pH range at which acidification is stopped in the case of a conventional method), and stabilizes, even if the milk is maintained at fermentation temperature, at a minimum pH. The value of this minimum pH depends essentially on the amount of glucose added.

This property makes it possible to reduce, or even to eliminate, the cooling phase used in conventional methods for manufacturing yogurt to stop the fermentation. It also eliminates the necessity of measuring the pH to determine the optimum moment for stopping the fermentation; for a given ferment and amount of added glucose, it is possible, without risk of overacidification, to stop the fermentation at the end of a given period, calculated as a function of the time required to reach the minimum pH. This makes it possible to have better control of the regularity of the final pH and of the texture for the product at the end of fermentation.

Advantageously, for the implementation of the method in accordance with the invention, and depending on the degree of acidification that it is desired to reach, the amount of glucose added to the milk prior to the fermentation is between 0.5 and 10 g/l, preferably between 0.5 and 5 g/l.

The fermented product obtained in this way can be conserved for several hours at a temperature close to the fermentation temperature, without a drop in pH, thereby making it possible to eliminate the installations for intermediate cold storage, and to increase the capacity of the fermentation tanks.

The implementation of the method in accordance with the invention makes it possible to reduce the postacidification in the fermented products during their longer term conservation. The degree of post-

acidification can vary depending on the composition of the ferment and the amount of glucose used. However, the postacidification is always clearly lower than that observed in the case of yogurts obtained with conventional ferments and methods.

For example, experiments carried out by the inventors have shown that, under the same conservation conditions (28 days of conservation at 10°C), the ΔpH (difference between the pH at D0 and the pH at D28) is between 0.05 and 0.4 in the case of the products obtained using a ferment in accordance with the invention, whereas it is always greater than 0.7 in the case of control ferments in which the strain of *L. bulgaricus* in accordance with the invention is replaced with a wild-type strain.

This weak postacidification is accompanied by good survival of the strains of the ferment; the population of *L. bulgaricus*, at the end of conservation, in the fermented product obtained in accordance with the invention is only slightly smaller than that of the control product.

A subject of the present invention is also the fermented dairy products which can be obtained by implementing a method in accordance with the invention.

These products can be conserved for a longer time and at higher temperatures than the products obtained using conventional methods, and have organoleptic properties which remain stable during conservation.

EXAMPLE 1: BIOCHEMICAL ASSAYING OF THE BETA-GALACTOSIDASE ACTIVITY OF A MUTANT IN ACCORDANCE WITH THE INVENTION

The β -galactosidase activity of the I-1968 strain was compared with that of the wild-type strain of *L. bulgaricus* (hereafter termed LbS) from which it is derived.

The bacteria are cultured overnight on MRS agar medium (MERCK) at 37°C, in an anaerobiosis jar (MERCK)

in the presence of an oxygen fixer (AnaerocultA, MERCK).

5 A 10-microliter loop (NUNC) of bacteria is resuspended in 1 milliliter of sterile water. The bacteria are lyzed with 2 cycles of vigorous shaking, 20 seconds at 5000 rotations per minute in the presence of glass microbeads (0.5 mm in diameter, BIOSPEC PRODUCTS), and then addition of 0.15 ml of chloroform. The mixture is shaken for 30 minutes at 37°C, and the volume is made to 2 ml with sterile water at 4°C. The beta-galactosidase activity is then measured: starting with 0.2 ml of the cell suspension, 1.2 ml of 0.067M NaH₂PO₄ buffer, pH 6.8; 0.05 ml of L-cysteine (SIGMA) at 10 t0 0.05 ml of O-nitrophenyl-beta-D-galactopyranoside (SIGMA) are added. The enzymatic reaction is stopped after 0, 2, 5 or 10 min, with 1 ml of 10% Na₂CO₃ buffer, and, after centrifugation of the reaction medium, a measurement of the OD at 400 nanometers is performed on the supernatant.

20 The galactosidase activities of the LbS parent strain and of the I-1968 mutant in accordance with the invention, measured as a function of time, are given in Figure 1.

25 These results show that the β -galactosidase is totally inactive in the mutant in accordance with the invention.

EXAMPLE 2: STABILITY OF THE I-1968 MUTANT OF *L. BULGARICUS*

30 The stability of the I-1968 mutant was tested in media containing, as carbon sources, either a mixture of glucose and of lactose, or lactose only.

35 An I-1968 culture obtained on MRS medium containing glucose is subcultured on sterilized milk which is supplemented with yeast autolyzate (2 g/l) and which may or may not be supplemented with glucose (20 g/l). When a pH of 5.2 (coagulation of the milk) is reached, samples of each subculturing are taken, on which the capacity of the bacteria to ferment sugars, as well as the presence of β -galactosidase activity

(X-gal plate assay: white colonies = β -galactosidase minus; blue colonies = β -galactosidase plus), and analyzed.

The results are given in Table 1 below.

5

TABLE I

Medium	Milk + glucose (20 g/l)	Milk
Time to reach pH 5.2	6h00	20h00
Fermentation of sugars	glucose, fructose, mannose	lactose, glucose, fructose, mannose
X-gal plate assay	100% white colonies	20% white colonies 80% blue colonies

These results show that, in the presence of glucose, the I-1968 strain does not revert toward a strain capable of using lactose. Conversely; in a medium containing lactose as the only carbon source, rapid reversion of the I-1968 strain toward the original state is observed.

EXAMPLE 3: ACIDIFICATION, POSTACIDIFICATION AND SURVIVAL PROPERTIES OF THE I-1968 VARIANT OF *L. BULGARICUS* IN SYMBIOSIS WITH *S. THERMOPHILUS*: THE CASE OF A METHOD FOR MANUFACTURING A SET YOGURT (FERMENTATION IN A VENTILATED OVEN)

Yogurt ferments are prepared combining the I-1968 strain in accordance with the invention with various industrial strains of *S. thermophilus* (the strains of *S. thermophilus* used are hereafter termed ST1, ST2 and ST3).

By way of comparison, the ferments are prepared combining the LbS parent strain and the same strains of *S. thermophilus*.

For preparing the ferments, the strains are seeded separately and at 1% on the following composition:

Composition for 1 liter:

135 g of skimmed milk powder
2 g of yeast autolyzate
920 ml of distilled water
20 g of glucose (for the I-1968 strain only)
Hydration: 10 min

101120 4890263

Pasteurization: 30 min at 95°C

The milk is then cooled to 44°C and inoculated, and then incubated at 44°C until an acidity of 85°D (degrees Dornic) for the streptococci and of 80°D for the lactobacilli is obtained.

The cultures are then cooled so as to obtain a ferment consisting of 80% *Streptococcus thermophilus* and of 20% *Lactobacillus bulgaricus*.

The ferments thus obtained are used to inoculate the following preparation:

Composition for 1 liter:

99% of milk

0, 1, or 2 g/l of glucose

Hydration: 10 min

15 Pasteurization: 10 min at 95°C

The milk is then cooled to 44°C and inoculated at 1%.

For each experiment, the composition of the ferment and the amount of glucose added are given in Table II below:

TABLE II

Experiment	Glucose g/l	Strains	Percentage
1	0	ST 3	64%
		ST 2	16%
		LbS	20%
2	0	ST 3	64%
		ST 2	16%
		I-1968	20%
3	1	ST 3	64%
		ST 2	16%
		I-1968	20%
4	0	ST 1	80%
		LbS	20%
5	0	ST 1	80%
		I-1968	20%
6	2	ST 1	80%
		I-1968	20%

After inoculation, the milk is distributed into round-bottomed flasks and incubated at a temperature of 44°C. The acidification profile is monitored during the incubation. The products are uncureddled at pH 4.6 by cooling in a cold unit (16 hours at 4°C).

The products are then subjected to a conservation test at 10°C. In this test, the pH and Dornic acidity are measured after 1, 14, 21 and 28 days of conservation.

- 5 The acidification results (time to reach a pH of 4.6 and pH value at 24 h) are given in Table III below:

TABLE III

Experiment	Time to reach pH 4.6 (min)	Time to reach pH 4.5 (min)	pH at 24 h
1	215	236	3.67
2	550	778	4.33
3	416	507	4.26
4	225	241	3.67
5	660	>1500	4.54
6	390	465	4.35

- 10 The results of the conservation test at 10°C (monitoring of the pH and of the Dornic acidity) and the survival test (*S. thermophilus* and *L. bulgaricus* populations) at 28 days are given in Table IV below:

TABLE IV

Experiment	Storage time (days)	pH	Dornic acidity	<i>Streptococcus thermophilus</i> cells/ml	<i>Lactobacillus bulgaricus</i> cells/ml
1	1	4.41	101	7.25E+08	3.35E+08
1	14	3.98	140	ND	ND
1	21	3.95	145	ND	ND
1	28	3.9	148	7.35E+08	3.30E+08
2	1	4.5	93	5.60E+08	2.90E+07
2	14	4.23	110	ND	ND
2	21	4.18	112	ND	ND
2	28	4.19	114	5.65E+08	1.87E+07
3	1	4.49	96	6.90E+08	7.45E+07
3	14	4.14	115	ND	ND
3	21	4.15	117	ND	ND
3	28	4.15	120	8.65E+08	6.30E+07
4	1	4.39	105	6.30E+07	4.40E+08
4	14	3.91	145	ND	ND
4	21	3.9	151	ND	ND
4	28	3.85	157	4.70E+08	6.30E+08
5	1	4.6	85	9.05E+08	6.70E+07
5	14	4.58	80	ND	ND
5	21	4.53	80	ND	ND
5	28	4.61	79	9.40E+08	7.00E+07

Experiment	Storage time (days)	pH	Dornic acidity	<i>Streptococcus thermophilus</i> cells/ml	<i>Lactobacillus bulgaricus</i> cells/ml
6	1	4.51	89	1.05E+09	1.96E+08
6	14	4.38	90	ND	ND
6	21	4.39	96	ND	ND
6	28	4.42	90	1.62E+09	1.91E+08

ND = Not Determined

These results show that the yogurts produced using the symbioses combining the I-1968 strain with one or two strains of *S. thermophilus* show extremely
 5 reduced postacidification with respect to the same symbioses with the LbS parent strain, while at the same time conserving an abundant population at the end of fermentation and good survival for 28 days at 10°C.

Stopping the acidification and maintaining the
 10 pH at around 4.6 to 4.5 for at least 24 hours at 44°C makes it possible, in the context of manufacturing stirred yogurt, to reduce or even eliminate the phase of cooling in a tank, which is conventionally used.

INDICATIONS RELATIVES À UN MICRO-ORGANISME OU
AUTRE MATÉRIEL BIOLOGIQUE DÉPOSÉ

(règle 13bis du PCT)

A. Les indications ont trait au micro-organisme ou autre matériel biologique visé dans la description page <u>4</u> , ligne <u>9-22</u>	
B. IDENTIFICATION DU DÉPÔT D'autres dépôts font l'objet d'une feuille supplémentaire <input type="checkbox"/>	
Nom de l'institution de dépôt Collection Nationale de Cultures de Micro-organismes	
Adresse de l'institution de dépôt (y compris le code postal et le pays) 28 rue du Docteur Roux, 75724 PARIS CEDEX 15, FRANCE	
Date du dépôt January 14, 1998	n° d'ordre I-1968
C. INDICATIONS SUPPLÉMENTAIRES (le cas échéant) Une feuille supplémentaire est jointe pour la suite de ces renseignements <input type="checkbox"/>	
"With regard to the designations under which a European patent is requested, a sample of the microorganism deposited will be accessible, up to the publication of the mention of the grant of the European patent or up to the date on which the application is rejected, withdrawn or deemed to be withdrawn, only through the handing over of a sample to an expert designated by the Applicant (Rule 28.4 of the EPC)".	
D. ÉTATS DESIGNÉS POUR LESQUELS LES INDICATIONS SONT DONNÉES <i>(si les indications ne sont pas données pour tous les États désignés)</i>	
ALL THE PCT MEMBER COUNTRIES	
E. INDICATIONS FOURNIES SÉPARÉMENT (le cas échéant)	
Les indications énumérées ci-après seront fournies ultérieurement au Bureau international (spécifier la nature générale des indications p. ex., "n° d'ordre du dépôt")	

Réservé à l'office receveur <input checked="" type="checkbox"/> Cette feuille a été reçue en même temps que la demande internationale Fonctionnaire autorisé [illegible signature]	Réservé au Bureau international <input type="checkbox"/> Cette feuille est parvenue au Bureau international le : Fonctionnaire autorisé
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CLAIMS

1. A mutant strain of *L. bulgaricus* lacking
5 β -galactosidase activity, characterized in that it carries a non-sense mutation in at least one of the coding sequences of the lactose operon.
2. The mutant strain of *L. bulgaricus* as claimed in claim 1, characterized in that said coding sequence
10 is the sequence encoding β -galactosidase.
3. The mutant strain of *L. bulgaricus* as claimed in either of claims 1 and 2, deposited on January 14, 1998 with the CNCM under the number I-1968.
4. A lactic ferment, characterized in that it
15 comprises at least one strain of *L. bulgaricus* as claimed in any one of claims 1 to 3.
5. The lactic ferment as claimed in claim 4, characterized in that said strain of *L. bulgaricus* is combined with at least one strain of *S. thermophilus*.
- 20 6. A method for preparing a fermented dairy product, characterized in that it comprises a step during which milk is fermented using a lactic ferment comprising at least one strain of *L. bulgaricus* as claimed in any one of claims 1 to 3, in the presence of
25 at least one sugar which can be assimilated by said strain.
7. The method as claimed in claim 6, characterized in that said sugar which can be assimilated is glucose.
8. The method as claimed in either of claims 6
30 and 7, characterized in that the arrest of fermentation is carried out without cooling of said dairy product.
9. A fermented dairy product which can be obtained using a method as claimed in any one of claims 6 to 8.
10. The fermented dairy as claimed in claim 9,
35 characterized in that said product is a yogurt.

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SEQUENCE LISTING

<110> COMPAGNIE GERVAIS DANONE
GENDRE, François
BENBADIS, Laurent
BRIGNON, Pierre

<120> MUTANT LACTOBACILLUS BULGARICUS STRAINS FREE
FROM BETA-GALACTOSIDASE ACTIVITY

<130> MJPCb191/143

<140>

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<150> FR9806456

<151> 1998-05-22

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Ser Asn Pro Val Val Tyr Leu Val Ile Phe Gly Ile Val Tyr Leu Ile
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Asp Ser Asn Phe Lys Ser Val Lys Val Pro Gly Asn Leu Glu Leu Gln
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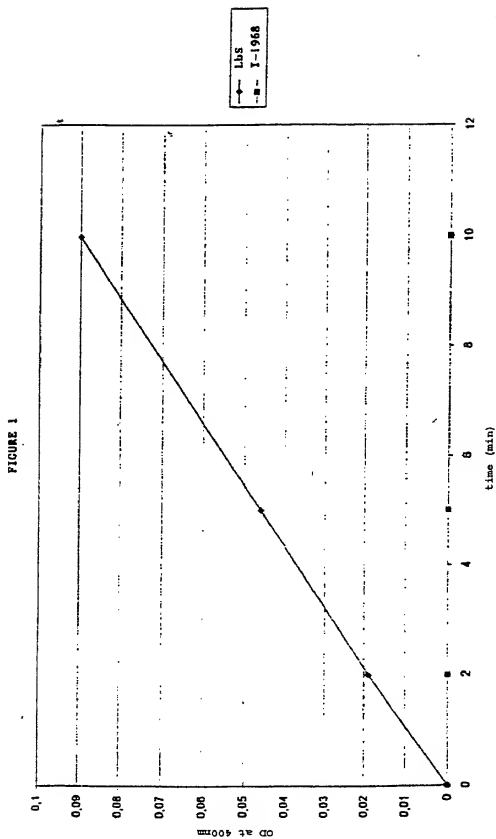
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Lys	Glu	Leu	Thr	Asp	Tyr	Arg	Tyr	Tyr	Gly	Leu	Gly	Pro	Asn	Glu	Ser
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French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119(a)-(d) ou § 365(b) du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur ou, en vertu du Titre 35, § 365(p) du même Code, sur toute demande internationale PCT désignant au moins un pays autre que les Etats-Unis et figurant ci-dessous et, en cochant la case, j'ai aussi indiqué ci-dessous toute demande étrangère de brevet, tout certificat d'inventeur ou toute demande internationale PCT ayant date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

Prior Foreign application(s)

Demande(s) de brevet antérieure(s) dans un autre pays.

FR 98 06456

(Number) (Country)
(Numéro) (Pays)

(Number) (Country)
(Numéro) (Pays)

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 119(e) du Code des Etats-Unis, de toute demande de brevet provisoire effectuée aux Etats-Unis et figurant ci-dessous.

(Application No.) (Filing Date)
(N° de demande) (Date de dépôt)

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis, ou en vertu du Titre 35, § 365(d) du même Code, de toute demande internationale PCT désignant les Etats-Unis et figurant ci-dessous et, dans la mesure où l'objet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande américaine ou internationale PCT, en vertu des dispositions du premier paragraphe du Titre 35, § 112 du code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la brevetabilité, comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la demande antérieure et la date de dépôt de la demande nationale ou internationale PCT de la présente demande :

(Application No.) (Filing Date)
(N° de demande) (Date de dépôt)

(Application No.) (Filing Date)
(N° de demande) (Date de dépôt)

Je déclare que par le présent acte qu'une déclaration ci-incluse est, à ma connaissance, véritable et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la section 1001 du Titre 18 du Code des Etats-Unis, et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hereby claim foreign priority under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(p) of any PCT International application which designated at least one country other than the United States, listed below, and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Priority claimed Droit de priorité revendiqué

May 22, 1998

(Day/Month/Year Filed)
(Jour/Mois/Année de dépôt)

☒ Yes
Oui

☐ No
Non

(Day/Month/Year Filed)
(Jour/Mois/Année de dépôt)

☐ Yes
Oui

☐ No
Non

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application No.) (Filing Date)
(N° de demande) (Date de dépôt)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Status) (patented, pending, abandoned)
(Statut) (breveté, en cours d'examen, abandonné)

(Status) (patented, pending, abandoned)
(Statut) (breveté, en cours d'examen, abandonné)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocat(s) et/ou agent(s) suivant(s) pour qu'ils poursuive(nt) la procédure de cette demande de brevet, et traite(nt) toute affaire s'y rapportant avec l'Office des brevets et des marques : (mentionner le nom et le numéro d'enregistrement).

POWER OF ATTORNEY : As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith : (list name and number)

All practitioners associated with
CUSTOMER NUMBER 000826

RAYMOND O. LINKER, JR. Registration No. 26,419

Adresser toute correspondance à :

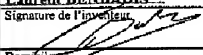
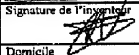
Send Correspondence to :

ALSTON & BIRD LLP
311 East Morehead Street
P.O. Drawer 34009
CHARLOTTE, NC 28234-4009 U.S.A.

Adresser tout appel téléphonique à :
(nom et numéro de téléphone)

Direct Telephone calls to : (name and telephone number)

(704) 331-6000

Nom complet de l'unique ou premier inventeur Laurent BENRADIS		Full name of sole or first inventor	
Signature de l'inventeur 	Date 1/2/00	Inventor's signature	Date
Domicile F-92160 Anthony (FRANCE)	FRX	Residence	
Nationalité Française		Citizenship	
Adresse Postale 7, avenue de Provence F-92160 Anthony (FRANCE))		Post Office Address	
Nom complet du second co-inventeur, le cas échéant Pierre BRIGNON		Full name of second joint inventor, if any	
Signature de l'inventeur 	Date 27/11/00	Second inventor's signature	Date
Domicile F-67200 Strasbourg (FRANCE)	FRX	Residence	
Nationalité Française		Citizenship	
Adresse Postale 7, rue des Brasseurs F-67200 Strasbourg (FRANCE)		Post Office Address	

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

(Supply similar information and signature for third and subsequent joint inventors.)

French Language Declaration

Nom complet du troisième co-inventeur, le cas échéant François G. DRE		Full name of third joint inventor, if any	
Signature de l'inventeur	Date 4/12/00	Third inventor's signature	Date
Domicile P-67200 Strasbourg (FRANCE)	FRX	Residence	
Nationalité Française		Citizenship	
Adresse Postale 49, rue du Maréchal Foch P-67200 Strasbourg (FRANCE)		Post Office Address	
Nom complet du quatrième co-inventeur, le cas échéant		Full name of fourth joint inventor, if any	
Signature de l'inventeur	Date	Fourth inventor's signature	Date
Domicile		Residence	
Nationalité Française		Citizenship	
Adresse Postale		Post Office Address	
Nom complet du cinquième co-inventeur, le cas échéant		Full name of fifth joint inventor, if any	
Signature de l'inventeur	Date	Fifth inventor's signature	Date
Domicile		Residence	
Nationalité		Citizenship	
Adresse Postale		Post Office Address	
Nom complet du sixième co-inventeur, le cas échéant		Full name of sixth joint inventor, if any	
Signature de l'inventeur	Date	Sixth inventor's signature	Date
Domicile		Residence	
Nationalité		Citizenship	
Adresse Postale		Post Office Address	

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

Supply similar information and signature for third and subsequent joint inventors.)